ELSEVIER

Journal of Virological Methods 111 (2003) 75–84

Journal of Virological Methods

www.elsevier.com/locate/jviromet

Shuttle PCR-based cloning of the infectious adeno-associated virus type 5 genome

Kyuhyun Lee, Young-Gun Kim, Eui-Cheol Jo *

MOGAM Biotechnology Research Institute, 341 Pojung-Ri Koosung-Eup, Yongin, Kyonggi-Do 449-913, South Korea

Received 26 December 2002; received in revised form 10 April 2003; accepted 10 April 2003

Abstract

Adeno-associated virus type 5 (AAV5), which is distinct from the other serotypes of AAV, has attracted considerable interest as a premier gene delivery vector. As do the other serotypes, AAV5 contains its 4.7 kb-sized, single-stranded genome flanked with inverted terminal repeats (ITRs) in a hairpin conformation, which serves frequently as pause and arrest sites for DNA polymerases during PCR. To amplify the full-length of the AAV5 genome in single step, we established a shuttled, long and accurate PCR (LA-PCR) procedure in the present study. Furthermore, helper oligonucleotides, which hybridize with the palindromic sequence elements in ITR, were designed and employed in PCR to prevent the formation of hairpin structures by highly GC-rich ITRs. Consequently, a 4.7 kb-sized PCR product was amplified successfully, and cloned into a pBluescript® II KS(+) plasmid. Six plasmids, harboring the full-length AAV5 genome, rescued wild type AAV5 viruses on transfection to HeLa and HEK 293 cells, which were co-infected with helper adenoviruses. Western and Southern blot analyses supported further the fact that the pAAV5 plasmids harbored the full-length AAV5 genome. The PCR method described in this study is applicable for the cloning of genomes containing variable palindromic structures, in addition to AAV genomes of other serotypes.
Published by Elsevier B.V.

Keywords: Adeno-associated virus type 5; Shuttle-PCR; Helper oligonucleotide; Infectious clone; LA-PCR

1. Introduction

Adeno-associated virus (AAV) is a small, non-enveloped, non-pathogenic and single-stranded DNA virus. The generation of infectious AAV plasmids has made it possible to explore the life cycle of AAV and to develop promising gene therapy vectors (Bueler, 1999; Monahan and Samulski, 2000). Although genomic sequences of the AAVs of eight different primates have been identified, the most popular gene therapy vectors have been derived from the serotype 2 (AAV2). Of other serotypes of AAV, AAV5 has the least homologous DNA sequence to AAV2 (Bantel-Schaal et al., 1999), and its transduction is not sensitive to heparin, which inhibits AAV2 (Chiorini et al., 1999). These relationships suggest that AAV5 enters cells by using different receptors from those used by AAV2, and thus shows a peculiar tissue tropism (Zabner et al., 2000; Walters et al., 2001). The AAV5-derived vectors were found to be more efficient than AAV2-derived vectors for gene transfer to muscle, liver and ependymal cells in the cerebral ventricles and hemispheres (Davidson et al., 2000; Chao et al., 2000; Mingozzi et al., 2002). In addition, hybrid vectors based on AAV2 and AAV5 exhibit high transduction efficiencies to muscle, retina and airway epithelial cells (Rabinowitz et al., 2002; Hildinger et al., 2001; Duan et al., 2001). Furthermore, the fact that neutralizing antibody to AAV5 was not detected in human population very much favors the development of a gene transfer vector based on AAV5 (Hildinger et al., 2001).

AAV contains a 4.7 kb-sized, single-stranded genome with inverted terminal repeats (ITRs) in the hairpin conformation, but during its life cycle, it generates many different genomic intermediates (Xiao et al., 1997; Wang et al., 1997). All direct molecular cloning with the AAV genome starts with either single-stranded viral DNA or double-stranded intermediate, and this leads to a string

* Corresponding author. Tel.: +82-31-260-9849; fax: +82-31-260-9808.
E-mail address: ecjo@mogam.re.kr (E.-C. Jo).

0166-0934/03/$ - see front matter. Published by Elsevier B.V.
doi:10.1016/S0166-0934(03)00135-6

of steps involving the annealing of complementary single strands, end-filling, attaching linkers, selecting a lower-copy number plasmid, blunt-end ligation, colony selection through hybridization, and finally reassembling subgenomic fragments (Chiorini et al., 1999; Samulski et al., 1982; Laughlin et al., 1983; Hermonat and Muzyczka, 1984; Xiao et al., 1999; Muramatsu et al., 1996; Chiorini et al., 1997; Qiu et al., 2002). Many of these step manipulations take time and infrequently generate positive results, and thus, require elaborate efforts to obtain the final infectious clone often at a probability of less than 10% (Samulski et al., 1982; Senapathy and Carter, 1984). Such attempts have experienced difficulties in terms of isolating full-length infectious AAV clones in a single step, in fact infectious viral genomes have been usually obtained by reassembling cloned subgenomic fragments (Laughlin et al., 1983; Xiao et al., 1999; Muramatsu et al., 1996; Chiorini et al., 1997; Qiu et al., 2002).

The polymerase chain reaction (PCR) is also a powerful tool for the amplification of genetic sequences, and it is an alternative way of direct molecular cloning (Cheng et al., 1994). With the advent of powerful DNA polymerases, the length of amplifiable target DNA exceeds 5–6 kb and further spans to 35 kb. As far as length is concerned, the 4.7 kb-sized genome of AAV5 is within the scope of target DNA that advanced *Taq* DNA polymerases can amplify (Barnes, 1994).

The first 137 nucleotides at both ends of the ITR region, the only *cis*-acting element necessary for the rescue of AAV5, are highly GC-rich and contain a palindromic structure. This secondary structure folds back on itself to form a T-shaped hairpin structure (Ferrari et al., 1996), which impedes the progression of a DNA polymerase even at the elevated temperatures employed in the PCR procedures (Potaman and Bissler, 1999). A common way of overcoming this structural barrier involves the addition of denaturants, such as dimethylsulfoxide (DMSO), betaine, low molecular weight sulfones, and nucleotide analogues (Cusi et al., 1992; Henke et al., 1997; Chakrabarti and Schutt, 2001). These molecules destabilize secondary structures in the template, and thereby enhance polymerization efficiency. Beyond doubt, the pivotal point is how to hold a GC-rich template, prone to form a hairpin structure, to its linear state during PCR. An oligonucleotide that hybridizes with the template and prevents its folding can be exploited to minimize this structural barrier for DNA polymerization (Potaman and Bissler, 1999).

To clone the full-length AAV5 genome, we performed the following; (i) we surveyed DNA polymerases that can support the synthesis of the full genome of AAV in single-step, (ii) we designed and employed candidate helper oligonucleotides, which can prevent hairpin structure formation during PCR, and (iii) we developed a shuttle PCR protocol to enhance the fidelity of the amplified products. Here we describe an efficient way of cloning the full AAV5 genome by single-step PCR, which may be applicable to the cloning of various genomes containing palindromic structures and of the AAV genomes of other serotypes.

2. Materials and methods

2.1. Cell cultures and viruses

HeLa S3 and HEK 293 cells (CCL-2.2 and CRL-1573, ATCC, Rockville, MD, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Biowhittaker, Wakersville, MD, USA) at 37 °C in a humidified 5% CO_2 atmosphere. AAV serotype 5 (a gift from Dr. Ursula Bantel-Schaal at DKFZ, Heidelberg, Germany) and adenovirus type 2 (VR-846, ATCC) were grown and assayed in HeLa cells as described in detail elsewhere (Bantel-Schaal and zur Hausen, 1984). AAV5 was produced and isolated by CsCl gradient banding and fractionated according to a standard purification procedure (Bantel-Schaal and zur Hausen, 1984). Alternatively, the viral particles in the lysate supernatant was purified by iodixanol-gradient ultracentrifugation as described in detail elsewhere (Zolotukhin et al., 2002).

2.2. DNA amplification and cloning

Viral DNA was isolated from wild-type virus stocks using a NucleoSpin® Viral DNA preparation kit (Clontech, Palo Alto, CA, USA). Single primer, (5′-gctctagagCTCTCCCCCCTGTCGCGTTCGCTCGCTCGC-3′) active in both directions for the amplification of single-stranded AAV template and helper oligonucleotide I (5′-GAGCTGCCACGACGGCC-amine-3′), helper oligonucleotide II (5′-CGTCGCCCCCCCAAACGAGCCAGCGAGCGAGCGAACGCGA-amine-3′) and helper oligonucleotide III (5′-CCAAACGAGCCAGCGAGCGAGCGAACGCGACAGGGGGamine-3′) were custom synthesized by Genotech (Daejon, Korea). Lowercase bases represent overhangs that include sequences complementary to cloning recognition site for *Xba*I restriction endonuclease. Various DNA polymerases, *Ex Taq*™, *LA Taq*™ (Takara, Otsu, Japan), *KlenTaq*1™ (Ab Peptides, St. Louis, MO, USA), *AmpliTaq* Gold® (Applied Biosystems, Foster City, CA, USA), *PfuTurbo*®, *Taq* and *Taq* Extender™ (Stratagene, La Jolla, CA, USA), were used to amplify the AAV5 genome. The LA-16 enzyme mixture was prepared by mixing one volume of *KlenTaq*1™ with a sixteenth of this volume of *Pfu* DNA polymerase as described previously (Cheng et al., 1994; Barnes, 1994). PCR mixtures contained a total volume of 50 μl : 5 μl of

10 × PCR buffer optimal for each DNA polymerase, 2.5 U of DNA polymerase, 4 μl of 250 μM each dNTP, 250 nM helper oligonucleotide, 100 ng of the single-stranded viral DNA as template, and sterile deionized water to volume. Instead of usual PCR buffer, 25 μl of 2 × GC I buffer was used, when PCR was carried out using *LA Taq*™ DNA polymerase, according to the manufacturer's recommendations. The cycling conditions of shuttle PCR were: 96 °C for 5 min, 35 cycles to shuttle between 96 °C for 30 s and 68 °C for 3 min 30 s, and 72 °C for 3 min to extend completely. The PCR was conducted using a RoboCycler® Gradient 96 Temperature Cycler according to the manufacturer's protocol (Stratagene). For cloning, 1 μl of the PCR products were gently mixed with 0.5 μl of pCR® 2.1-TOPO® vector (10 ng/μl) (Invitrogen, Carlsbad, CA, USA), 0.5 μl of salt solution (1.2 M NaCl and 0.06 M $MgCl_2$) and 1 μl of sterile deionized water. After incubating for 5 min at room temperature, the mixture was transferred to a competent *E. coli* strain DH5α and the transformants were spread onto LB agar plates containing ampicillin (100 μg/ml) and X-gal (100 μg/ml) for blue/white screening.

Two plasmids harboring overlapping subgenome of AAV5 were presumed to retain nt 1–4007, and nt 1089–4642 of AAV5 genome, respectively, as confirmed by the sequencing of both terminal sequences of each plasmid. These plasmids were named pAAV5/1089–4642 and pAAV5/1–4007, respectively. A 2.7 kb *Eco*RI fragment from pAAV5/1089–4642 spanning nt 1986–4642 was inserted into the *Eco*RI site of pBluescript® II KS (+) (Stratagene). This plasmid retained nt 2731–4642 of the AAV5 genome when restricted by *Bam*HI, and to this, a *Bam*HI fragment of pAAV5/1089–4642 spanning nt 1288–2731 was inserted. This plasmid retained nt 1871–4642 of AAV5 when restricted by *Spe*I/*Aat*II, and to this, a *Spe*I/*Aat*II fragment of pAAV5/1–4007 spanning nt 1–1871 was finally inserted, thus forming pAAV5 harboring the full-length AAV5 genome. Transformation of pAAV5 was conducted using the methods described above. Subsequently, positive clones were selected by restriction analysis, and analyzed further by partial sequencing of inserted AAV5 genome in both terminal directions. Cycle sequencing was performed by the fluorescent dye terminator method (ABI PRISM® Big Dye™ ready reaction terminator cycle sequencing kit) on an ABI PRISM® 310 genetic analyzer according to the manufacturer's procedure (Applied Biosystems).

2.3. DNA transfection, Southern blot analysis, and Western blot analysis

The DNA used in this study was prepared by using an Endo-free plasmid kit (Qiagen, Hilden, Germany). For transfection, approximately 3×10^6 HeLa cells were seeded into a 60-mm^2 dish a day before the transfection, and infected with a helper virus, adenovirus wild type 2 (wt Ad2), 4 h before the transfection. The culture was finally transfected with pAAV5 plasmids purified from positive clones (pAAV5-1 to -6) by using jetPEI™, according to the manufacturer's procedure (PolyPlus, Illkirch, France). After 2 days incubation at 37 °C in a humidified 5% CO_2 atmosphere, crude transfectant lysate were prepared, subjected to an 8% SDS-PAGE and then transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA). Anti-AAV5 polyclonal antibody (pab-10108, Orbigen, San Diego, CA, USA) was used as the primary antibody to detect AAV5 capsid proteins. The signal caused by the secondary antibody, anti-rabbit HRP-conjugated antibody (kpl, Gaithersburg, MD, USA) was detected using a SuperSignal® West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA). To identify the monomeric and dimeric forms of the replicative AAV5 genome, half-confluent HeLa cells were infected with wt Ad2 at an M.O.I. of 20 and transfected with pAAV5-1 to -6, respectively. A day after incubating at 37 °C in a humidified 5% CO_2 atmosphere, the cells were collected and Hirt DNA was extracted from the transfectants and fractionated on a 1% TAE agarose gel. It was then capillary-transferred to a nitrocellulose membrane, and hybridized with a ^{32}P-labeled probe to the AAV5 genome, which was prepared by using a Prime-It II® random primer labeling kit (Stratagene) and the *Xba*I-restricted AAV5 genome as template. Radioisotope labeling of this probe was conducted using [α-^{32}P]-dCTP (NEN, Boston, MA, USA). The hybridized membrane was then exposed to a phosphorimager (BAS 1000, Fujifilm, Tokyo, Japan), or to X-ray film (Kodak, Rochester, NY, USA) for autoradiography.

3. Results

Both termini of the AAV5 genome (ITR) contain a 71% GC-rich, palindromic sequence that folds back on itself to form a T-shaped, hairpin-like single-stranded structure. After infection, the 3′ end of the T-shaped ITR serves as a primer for DNA replication converting the single-stranded genome into a double-stranded template with a covalently closed hairpin at one end. The GC content in the rest of the genome, mostly encoding the *rep* and the *cap* genes, is about 54%. Therefore, the ITR region presents an obvious barrier for DNA polymerase action and for the single-step amplification of the full-length AAV genome. Furthermore, this region is so complementary to itself and it could cause priming competition during PCR.

To amplify the viral genome, standard three-temperature PCR was carried out (heating to 96 °C for 5 min; 30 cycles of 96 °C for 1 min, 52 °C for 1 min, and 72 °C for 3 min 30 s; followed by a final extension for 3 min). *LA*

Taq™ and *Ex Taq*™ DNA polymerases were used, but it did not give any sign of amplification (data not shown). To enhance DNA polymerization, a shuttle PCR strategy was undertaken. Because the theoretical melting temperature (T_m) of the primer used was high enough (more than 72 °C), it was speculated that two-temperature PCR, i.e. performing annealing and elongation together, should improve priming specificity. PCR was carried out to shuttle between 96 °C and a series of temperatures between 66 and 72 °C in increments of 2 °C, and the highest fidelity of the amplified product was obtained by shuttling from 96 to 68 °C (data not shown). Accordingly, the cycling conditions of shuttle PCR were found to be: 96 °C for 5 min, 35 cycles to shuttle between 96 °C for 30 s and 68 °C for 3 min 30 s, and 72 °C for 3 min to completely extend. Several DNA polymerases were used to amplify the AAV5 genome. Shuttle PCR using the LA-16 enzyme mixture, *Ex Taq*™ and *LA Taq*™ DNA polymerases, generated a few amplified products, respectively, while *Taq* (with or without *Taq* Extender®), *PfuTurbo*®, or *AmpliTaq* Gold® DNA polymerases gave no signal at all (Fig. 1A). The degree of DNA polymerization increased in the order of the LA-16 enzyme mixture, *Ex Taq*™ and *LA Taq*™ DNA polymerases, as judged by the length of longest amplified products. These three enzymes are actually mixtures of unique DNA polymerases that perform Long and Accurate PCR (LA PCR). In particular, *LA Taq*™ polymerase gave rise to a 4.7 kb-sized band that was presumed to match the full-length AAV5 genome. The observed length of the major PCR products corresponded to 900 bp and 1.9 kb using the LA-16 mixture, and 1.9, 2.8 and 3 kb using *Ex Taq*™ DNA polymerase, and these DNA fragments were assumed to represent partially extended products of the template DNA. Assuming that this insufficient extension is associated with the structural barrier in ITR, we designed three helper oligonucleotides to hybridize to a region spanning the stem part to primer binding region in ITR (Fig. 1B). These three oligonucleotides modify 3′-OH to 3′-NH_2 on DNA and cannot prime DNA polymerization. For these reasons, they are believed to prevent hairpin structure formation, and furthermore, to reduce priming competition from template DNA. Using DNA polymerases in combination with these oligonucleotides, we performed DNA amplification. Contrary to our expectation, helper oligonucleotide I gave no signal of PCR amplification when run with the LA-16 mixture and *Ex Taq*™ DNA polymerase. However, helper oligonucleotide II and III allowed to generate two further-extended products including a 4.7 kb-sized product. This 4.7 kb product was presumed to represent a fully extended version of the full-length AAV5 genome (Fig. 1C and D). These helper oligonucleotides did not exhibit any additive effect on the PCR mediated by *LA Taq*™ DNA polymerase (Fig. 1E).

The 4.7 kb-sized products were identified specific to the AAV5 genome, as proven by a Southern blot analysis using a ^{32}P-labeled probe to the *cap* gene of the AAV5 genome (Fig. 1F). It was also noted that helper oligonucleotide I, as well as helper oligonucleotides II and III, allowed actually to generate a 4.7 kb-sized band specific to the AAV5 genome, although it looked as if it did not exhibit any additive effect on PCR when observed in an ethidium bromide-stained gel (Fig. 1C).

Consequently, each LA PCR generated a 4.7 kb-sized band presumed to correspond to the full-length AAV5 genome (Fig. 1). Further addition of betaine, DMSO, or TMA oxalate to these PCR did not help eliminate minor differential products or enhance the fidelity of the 4.7 kb-sized product generated by each LA PCR (data not shown). Therefore, each 4.7 kb-sized band was extracted from sliced gel for restriction analysis and for further cloning. The 4.7 kb-sized PCR product would be fragmented to 1038, 1321 and 2293 bp fragments by *Apa*I digestion, and 1678 and 2974 bp fragments by *Sal*I digestion, according to the restriction map deduced from the AAV5 genome sequence reported previously (GenBank accession no. AF085716). The sizes of the

Fig. 1. PCR amplification of the full-length AAV5 genome. (A) Shuttle PCR by various DNA polymerases. M: 1 kb plus DNA ladder (Life Technologies, Grand Island, NY, USA), lane 1: *Taq* DNA polymerase, lane 2: *Taq* DNA polymerase with *Taq* extender®, lane 3: *PfuTurbo*® DNA polymerase, lane 4: *AmpliTaq* Gold® DNA polymerase, lane 5: *Ex Taq*™ DNA polymerase, lane 6: LA-16 enzyme mixture and lane 7: *LA Taq*™ DNA polymerase. The arrow indicates the 4.7 kb-sized band presumed to correspond to the full-length AAV5 genome. (B) Schematic depiction of AAV5 ITR and the binding region of helper oligonucleotides. (C), (D), and (E) Additive effect of helper oligonucleotide I, II and III on shuttle PCR by LA-16 enzyme mixture, *Ex Taq*™ and *LA Taq*™ DNA polymerases, respectively. M: 1 kb plus DNA ladder (Life Technologies), lane 1: shuttle PCR without helper oligonucleotide, lane 2: shuttle PCR with helper oligonucleotide I, lane 3: shuttle PCR with helper oligonucleotide II, lane 4: shuttle PCR with helper oligonucleotide III. Helper oligonucleotides were added to the PCR mixture at a concentration of 250 nM. The arrows indicate the 4.7 kb-sized bands presumed to correspond to the full-length AAV5 genome. (F) Detection of the AAV5 genome by Southern blot analysis. Lane 1: shuttle PCR by *LA Taq*™ DNA polymerase without helper oligonucleotide, lane 2: shuttle PCR by *LA Taq*™ DNA polymerase with helper oligonucleotide II, lanes 3–6: shuttle PCR by LA-16 enzyme mixture without helper oligonucleotide, with helper oligonucleotide I, II and III, respectively, lane 7: the 4.7 kb-sized DNA isolated from the *Xba*I-restricted pAAV5 as a positive control. Each PCR product was capillary-transferred to a nitrocellulose membrane, and hybridized with a ^{32}P-labeled probe to the *cap* gene of the AAV5 genome, which was prepared by using a Prime-It II® random primer labeling kit (Stratagene) and the 1.4 kb-sized DNA isolated from the *Apa*I-restricted pAAV5 as template. The arrow indicates the 4.7 kb-sized bands presumed to correspond to the full-length AAV5 genome. Reverse contrast images were used to better visualize the effect of the PCR in Fig. 1A, C, D and E.

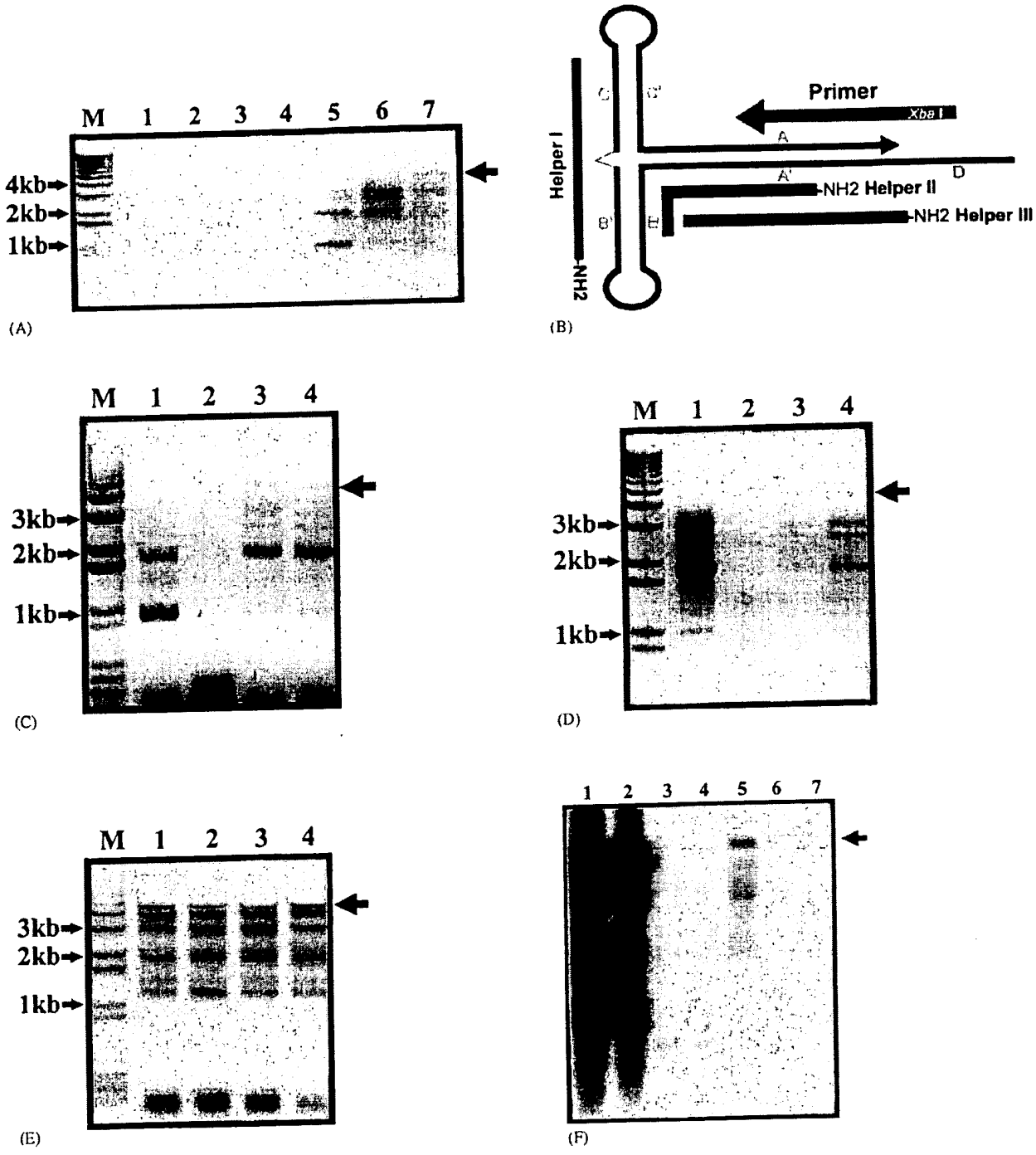


Fig. 1.

fragments generated were approximately 1.0, 1.4 and 2.2 kb for *Apa*I, and 1.7 and 3.0 kb for *Sal*I, which was in good agreement with the predicted sizes.

Topo cloning of the PCR reaction mixture was performed, and consequently, several clones harboring subgenomic fragments of the AAV5 genome were selected (data not shown). Recombining two overlapping subfragments assembled the AAV5 full genome in a pBluescript® II KS(+) backbone (Stratagene) and its transformation generated a hundred of colonies. Sixteen colonies were randomly selected, cultured, and the plasmids isolated from each clone were examined by *Bam*HI restriction mapping. According to the predicted plasmid map, the plasmid DNA would be fragmented to

212, 1443 and 5987 bp fragments by *Bam*HI digestion (Fig. 2A). All the plasmids isolated from the six clones (#3, #4, #7, #13, #14 and #15) generated 1.5 and 5.9 kb fragments, which was in good agreement with their predicted sizes (Fig. 2B). The predicted 212 bp fragment was probably eluted out of the gel because of its small size. Further restriction analysis of these six plasmids was carried out and this would generate 1089, 1321 and 5285 bp fragments by *Apa*I digestion, 345, 549, 1105, 2671 and 3025 bp fragments by *Eco*RI digestion, and 2966 and 4656 bp fragments by *Xba*I digestion. The sizes of the fragments generated by the six clones were 1.1, 1.4 and 5.2 kb for *Apa*I, 400, 500, 1.1, 2.7 and 3.0 kb for *Eco*RI, and 3.0 and 4.7 kb for *Xba*I (Fig. 2C, D and E). Restriction analysis by these endonucleases generated fragment sizes that agreed well with the predicted sizes. Additionally, these clones were confirmed by cycle sequencing. The sequencing skipped identifying 91 and 87 nucleotides in the stem part of the left and the right ITR, respectively. Skipped nucleotides were identified as nt 48–138 and nt 4509–4595 of the left and the right ITR, respectively, and they corresponded to the palindromic sequence elements C′, C, B′, B, and a part of A′. However, the remaining sequences in ITR (i.e. nt 1–47 and nt 139–524 in the left ITR, and nt 4642–4596 and nt 4508–4113 in the right ITR) were found to be identical to those reported previously (Chiorini et al., 1999). Because skipping of GC-rich ITR region usually occurs during cycle sequencing, it was hardly interpretable that these skipped regions had been deleted. As shown in Fig. 2D, restriction by *Eco*RI generated an approximately 500 bp fragment, which is generated only when the entire nucleotides constitutes the ITR without deletion. Instead of imperfect sequencing, this result supports the notion that these 91 and 87 nucleotides were not deleted (Fig. 2D). Therefore, it was assumed that these six plasmids contained the full-length AAV5 genome, and they were named pAAV5-1, pAAV5-2, pAAV5-3, pAAV5-4, pAAV5-5, and pAAV5-6, respectively.

Transfection of a plasmid harboring the full-sized AAV5 genome would generate intact AAV5 viruses in HEK 293 and HeLa cells, when co-infected with a helper virus such as wild-type adenovirus type 2 (Bantel-Schaal and zur Hausen, 1984). To examine this property, HeLa cells infected with wild-type adenovirus type 2 were transfected with each plasmid, pAAV5-1 to -6. A day post-transfection, Hirt DNA samples were extracted from the crude lysate of transfected cells and identified by Southern blot analysis using an AAV5-specific DNA probe. Both monomeric and dimeric forms of replicated AAV5 genome were detected in all the samples transfected with the six plasmids (Fig. 3, lanes 4–9), as was the case for the wild type AAV5 virus (Fig. 3, lane 3).

A further experiment was carried out on pAAV5-1 to -6 plasmids to examine their AAV5-specific capsid expression after transfection in HEK 293 cells. Western blot analysis was used to detect the capsid subunits VP1, -2, and -3 in crude lysates prepared from HeLa cells 48 h post-transfection. All the three capsid subunits VP1, -2, and -3 were detected in all the crude lysates by using anti-AAV5 polyclonal antibody (pab-10108, Orbigen), which recognizes amino acid residues 530–541 [NSQPANPGTTATC] of the capsid subunits of AAV5 (Fig. 4A). As deduced from the sequence analysis of the *cap* ORF (Bantel-Schaal et al., 1999; Chiorini et al., 1999), all the capsid subunits commonly contain these residues. Accordingly, anti-AAV5 antibody was found to detect all the capsid subunits of AAV5. From the same sequence analysis, the molecular masses of the VP1, -2, and -3 capsid proteins were predicted to be 80, 65 and 59 kDa, respectively (Bantel-Schaal et al., 1999), but these were all larger than the corresponding actual masses, as determined in this study. Besides, pAAV5-mediated capsid expression was obviously detected in all six crude lysates, suggesting that AAV5 rescued by the pAAV5-1 to -6 plasmids was adequately encapsidated.

To confirm that the virus particles in the lysate supernatant contain the AAV5 genome, they were purified by iodixanol-gradient ultracentrifugation, and subjected to Southern blot hybridization (Fig. 4B). The AAV5 containing fractions in the 40% iodixanol step gradient were collected and viral DNA from each fraction was isolated separately using a NucleoSpin® Viral DNA preparation kit (Clontech). It was capillary-transferred to a nitrocellulose membrane, and hybridized with a ^{32}P-labeled probe to the *cap* gene of the AAV5 genome. All the viral DNAs, isolated from the 40% density gradient, hybridized to the AAV5 specific probe, and generated the 4.7 kb-sized bands presumed to correspond to the full-length AAV5 genome.

Taken together, these results confirm that these pAAV5 plasmids harbor an infectious full-length AAV5 genome, and promote the rescue and replication of AAV5 viruses on transfection to wt Ad2-infected HeLa and HEK 293 cells.

4. Discussion

In this study, we demonstrate the successful generation of infectious plasmid clones of AAV5. Intact AAV5 DNA can be recovered, from these clones, by a single *Xba*I-cut for convenient use in various applications.

In terms of DNA length, the 4.7 kb-sized, AAV5 genome can be synthesized easily by several advanced DNA polymerases. However, the GC-rich 137 nucleotides at either end of the AAV5 genome are an obstacle to single step PCR amplification. This is evident from the result of the standard three-temperature and the two-temperature PCRs performed using various DNA polymerases. Both termini of the AAV5 genome are

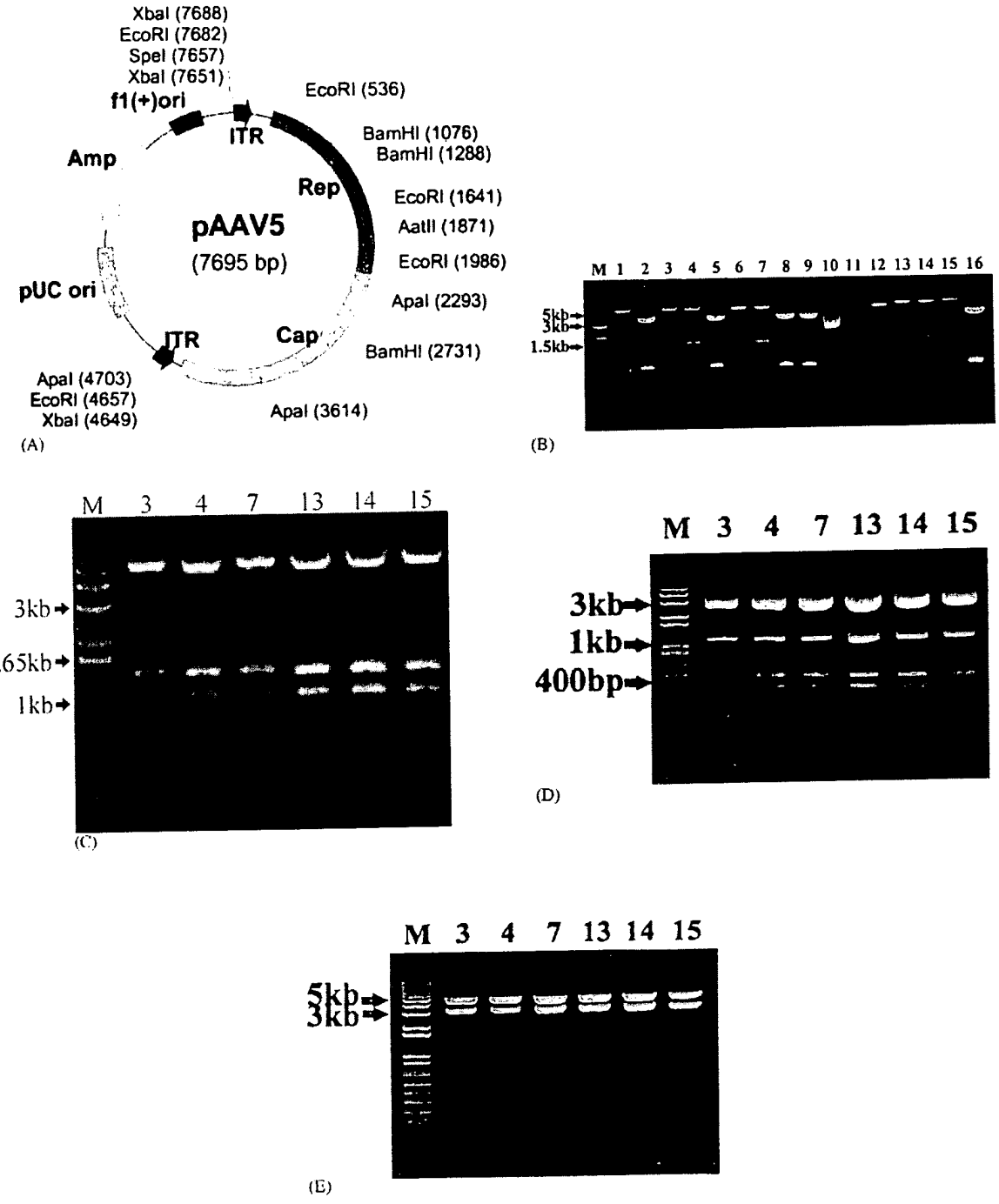


Fig. 2. Restriction analysis of positive clones harboring the AAV5 genome. (A) Vector map of pAAV5. (B) The *Bam*HI cleavage pattern of plasmid DNA extracted from white colonies. Plasmid DNAs were isolated from sixteen randomly selected colonies, and digested by *Bam*HI. These fragments were fractionated on a 1% agarose gel. M: 1 kb DNA ladder (NEB, Beverly, CA, USA). Lane numbers correspond to the numbers of each positive clone. (C), (D), and (E) Cleavage pattern of the plasmids, isolated from cultures of positive clones, by the restriction endonucleases, *Apa*I, *Eco*RI and *Xba*I, respectively. M: 1 kb plus DNA ladder (Life Technologies). Lane numbers correspond to the numbers of each positive clone.

71% GC-rich, but in the remainder of the genome, the GC content is about 54%. If the GC content varies within a long template, amplification can be greatly facilitated by two-temperature PCR (shuttle PCR) or sub-cycling PCR, as has been reported previously (Liu and Sommer, 1998). Although shuttle PCR amplified a few differential products only, this represented a substantial progress as compared to three-temperature

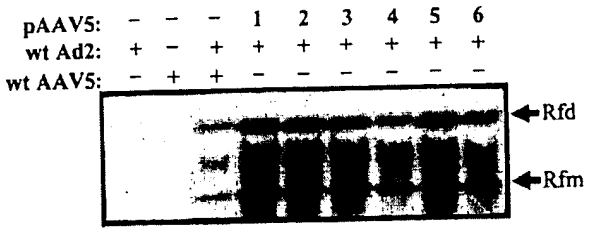
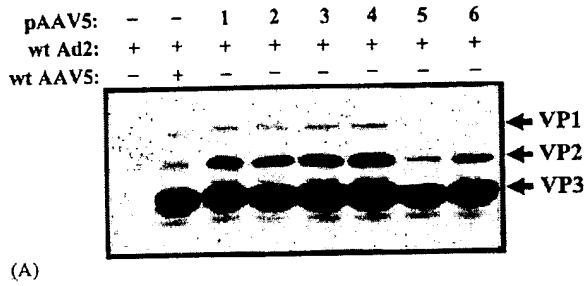


Fig. 3. Southern blot analysis of Hirt DNAs extracted from HeLa cells transfected with pAAV5-1 to -6. Hirt DNAs were isolated from cells collected 24 h post-transfection, digested with *Dpn*I to remove transfected DNA, electrophoresed on a 1% TAE-agarose gel, and analyzed by Southern blot using an AAV5-specific DNA probe. Rf_m and Rf_d denote the replicative monomeric and dimeric forms of the AAV5 genome, respectively.



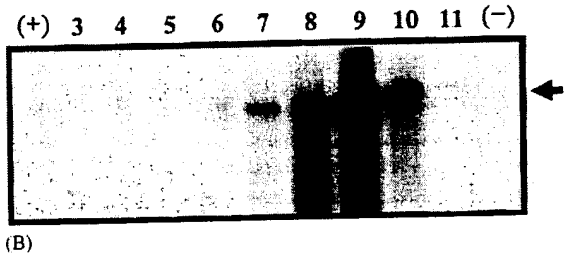


Fig. 4. Detection of the DNA-containing AAV5 particles. (A) Western blot analysis for identification of capsid subunits VP1, -2 and -3 of AAV5. The cells, collected and re-suspended in PBS 48 h post-transfection, were frozen and thawed three times, and then clarified by centrifugation. The supernatant was subjected to 8% SDS-PAGE. Polyclonal anti-AAV5 capsid antibody (Orbigen) was used at a dilution of 1:1000 as a primary antibody. (B) Southern blot analysis. The viral particles in the lysate supernatant were purified by iodixanol-gradient ultracentrifugation. Fractions in the 40% step gradient were collected from the bottom of the tube and viral DNA from each fraction was isolated separately using a NucleoSpin® Viral DNA preparation kit (Clontech). Southern blot hybridization was carried out using a probe specific to the *cap* gene of the AAV5 genome. Lanes marked 3–11: DNA samples isolated from each fraction. The numbers on the top of the gel correspond to the numbers of the fractions spanning from the 60 to 40% density junction to the 40 to 25% density junction. (+): DNA isolated from a CsCl gradient-purified wild type AAV5 virus stock, (–): DNA isolated from the crude lysate of HEK 293 cells infected with wt Ad2 only as a negative control.

PCR. The helper oligonucleotide allowed the perfect amplification of the full-length AAV genome by PCR with the LA-16 mixture and *Ex Taq*™ DNA polymerase. The ITR of AAV5 is a 167-bp element in which the first 137 nucleotides contain palindromic sequence elements in the order A, C′, C, B′, B, to A′ (Xiao et al., 1997; Wang et al., 1997). To prevent the single-stranded AAV molecules from folding, helper oligonucleotides I, II and III were designed to bind to the palindromic sequence elements A′, A′+B and C+B′, respectively. The additive effect of helper oligonucleotides II and III, which was shown weakly by helper oligonucleotide I (Fig. 1C, D and F), suggests that the binding of the palindromic sequence elements, A and A′ is more critical to the formation of hairpin structure of the ITR than either the B′/B, or C/C′ binding. Both helper oligonucleotides II and III, which contain complementary sequences to the palindromic sequence element A′, were indistinguishable in terms of their effect on amplification as observed on an ethidium bromide-stained gel (Fig. 1C and D). A corresponding Southern blot, however, showed that helper oligonucleotide II allowed to generate a more prominent 4.7 kb-sized band than helper oligonucleotide III did (Fig. 1F).

LA-PCR is based on the principle that a large amount of 3′-exonuclease-free thermostable DNA polymerase catalyzes the efficient extension of long target with accuracy, which is provided by the presence of minute quantities of DNA polymerase having 3′-editing exonuclease activity (Cheng et al., 1994; Barnes, 1994; Potaman and Bissler, 1999). The best combination was exhibited by combining *KlenTaq*1™ and *Pfu* DNA polymerase with an optimal ratio in the range 8–64. The LA-16 mixture, *Ex Taq*™ and *LA Taq*™ DNA polymerases resulted in the amplification of the full-length AAV5 genome in single step in the present study. All these DNA polymerases are actually unbalanced mixtures of two DNA polymerases, one lacking significant 3′-exonuclease activity and one exhibiting significant 3′-exonuclease activity. In particular, the LA-16 mixture and *Ex Taq*™ DNA polymerase, is thought to be the same formulation as produced by combining the two DNA polymerases, *KlenTaq*1™ and *Pfu* (Barnes, 1994). Accordingly, shuttle PCR using the LA-16 mixture and *Ex Taq*™ DNA polymerase exhibited almost the same results as shown in Fig. 1, and, with the aid of suitable helper oligonucleotides, generated a full-length AAV5 genome in single step. *LA Taq*™ DNA polymerase did not require the aid of helper oligonucleotides, which suggests the possibility that different pair of DNA polymerases constitute *LA Taq*™ DNA polymerase. If then, a more thermostable and accurate DNA polymerase might have replaced *Pfu* DNA polymerase in the formulation of *LA Taq*™ DNA polymerase. However, detailed information on *LA Taq*™ DNA polymerase is not currently available.

Until recently, genomic sequences of the AAV's of eight different primates have been identified, six types of AAV viruses have been isolated and six types of full-length infectious clones are available (Bantel-Schaal and

zur Hausen, 1984; Georg-Fries et al., 1984; Bantel-Schaal et al., 1999; Chiorini et al., 1999; Samulski et al., 1982; Laughlin et al., 1983; Xiao et al., 1999; Muramatsu et al., 1996; Chiorini et al., 1997; Qiu et al., 2002; Senapathy and Carter, 1984; Rutledge et al., 1998; Gao et al., 2002). As far as we are aware, the infectious plasmid pAAV5 is the first infectious clone known to harbor the AAV genome, which was amplified by PCR in a single step.

Shuttled LA PCR along with helper nucleotides not only facilitate cloning the AAV genome of the other serotypes, but will also expedite constructing active plasmids of full-length genomes containing various palindromic structures. The PCR-mediated cloning method of this study is simple, efficient and advantageous over the conventional direct molecular cloning methods that often involve lengthy experimental steps and rarely result in the generation of infectious clones.

Acknowledgements

We thank Dr. Ursula Bantel-Schaal at DKFZ (Heidelberg, Germany) for her kind gift of wt AAV5 stock. Part of this work was supported by the grant of Korean Ministry of Commerce, Industry and Energy (Grant Number: 00008099). We also deeply appreciate this support.

References

Bantel-Schaal, U., zur Hausen, H., 1984. Characterization of the DNA of a defective parvovirus isolated from a genital site. Virology 134, 52–63.

Bantel-Schaal, U., Delius, H., Schimidt, R., zur Hausen, H., 1999. Human adeno-associated virus type 5 is only distantly related to other known primate helper-dependent parvoviruses. J. Virol. 73, 939–947.

Barnes, W.M., 1994. PCR amplification of up to 35-kb DNA with high fidelity and high yield from λ bacteriophage templates. Proc. Natl. Acad. Sci. USA 91, 2216–2220.

Bueler, H., 1999. Adeno-associated viral vectors for gene transfer and gene therapy. Biol. Chem. 380, 613–622.

Chakrabarti, R., Schutt, C.E., 2001. The enhancement of PCR amplification by low molecular-weight sulfones. Gene 274, 293–298.

Chao, H., Liu, Y., Rabinowitz, J., Li, C., Samulski, R.J., Walsh, C.E., 2000. Several log increase in therapeutic transgene delivery by distinct adeno-associated viral serotype vectors. Mol. Ther. 2, 619–623.

Cheng, S., Fockler, C., Barnes, W., Higuchi, R., 1994. Effective amplification of long targets from cloned inserts and human genomic DNA. Proc. Natl. Acad. Sci. USA 91, 5695–5699.

Chiorini, J.A., Yang, L., Liu, Y., Safer, B., Kotin, R.M., 1997. Cloning of adeno-associated virus type 4 (AAV4) and generation of recombinant AAV4 particles. J. Virol. 71, 6823–6833.

Chiorini, J.A., Kim, F., Yang, L., Kotin, R.M., 1999. Cloning and characterization of adeno-associated virus type 5. J. Virol. 73, 1309–1319.

Cusi, M.G., Cioe, L., Rovera, G., 1992. PCR amplification of GC-rich templates containing palindromic sequences using initial alkali denaturation. Biotechniques 12, 502–504.

Davidson, B.L., Stein, C.S., Heth, J.A., Martins, I., Kotin, R.M., Derksen, T.A., Zabner, J., Ghodsi, A., Chiorini, J.A., 2000. Recombinant adeno-associated virus type 2, 4, and 5 vectors: transduction of variant cell types and regions in the mammalian central nervous system. Proc. Natl. Acad. Sci. USA 97, 3428–3432.

Duan, D., Yan, Z., Yue, Y., Ding, W., Engelhardt, J.F., 2001. Enhancement of muscle gene delivery with pseudotyped adeno-associated virus type 5 correlates with myoblast differentiation. J. Virol. 75, 7662–7671.

Ferrari, F.K., Samulski, T., Shenk, T., Samulski, R.J., 1996. Second-strand synthesis is a rate-limiting step for efficient transduction by recombinant adeno-associated virus vectors. J. Virol. 70, 3227–3234.

Gao, G.P., Alvira, M.R., Wang, L., Calcedo, R., Johnston, J., Wilson, J.M., 2002. Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. Proc. Natl. Acad. Sci. USA 99, 11854–11859.

Georg-Fries, B., Biederlack, S., Wolf, J., zur Hausen, H., 1984. Analysis of proteins, helper dependence, and seroepidemiology of a new human parvovirus. Virology 134, 64–71.

Henke, W., Herdel, K., Jung, K., Schnorr, D., Loening, S.A., 1997. Betaine improves the PCR amplification of GC-rich DNA sequences. Nucleic Acids Res. 25, 3957–3958.

Hermonat, P.L., Muzyczka, N., 1984. Use of adeno-associated virus as a mammalian DNA cloning vector: transduction of neomycin resistance into mammalian tissue culture cells. Proc. Natl. Acad. Sci. USA 81, 6466–6470.

Hildinger, M., Auricchio, A., Gao, G., Wang, L., Chirmule, N., Wilson, J.M., 2001. Hybrid vectors based on adeno-associated virus serotypes 2 and 5 for muscle-directed gene transfer. J. Virol. 75, 6199–6203.

Laughlin, C.A., Tratschin, J.D., Coon, H., Carter, B.J., 1983. Cloning of infectious adeno-associated virus genomes in bacterial plasmids. Gene 23, 65–73.

Liu, Q., Sommer, S.S., 1998. Subcycling-PCR for multiplex long-distance amplification of regions with high and low GC content: application to the inversion hotspot in the factor VIII gene. Biotechniques 25, 1022–1028.

Mingozzi, F., Schüttrumpf, J., Arruda, V.R., Liu, Y., Liu, Y.-L., High, K.A., Xiao, W., Herzog, R.W., 2002. Improved hepatic gene transfer by using an adeno-associated virus serotype 5 vector. J. Virol. 76, 10497–10502.

Monahan, P.E., Samulski, R.J., 2000. AAV vectors: is clinical success on the horizon? Gene Ther. 7, 24–30.

Muramatsu, S.-I., Mizukami, H., Young, N.S., Brown, K.E., 1996. Nucleotide sequencing and generation of an infectious clone of adeno-associated virus 3. Virology 221, 208–217.

Potaman, V.N., Bissler, J.J., 1999. Overcoming a barrier for DNA polymerization in triplex-forming sequences. Nucleic Acids Res. 27, e5.

Qiu, J., Nayak, R., Tullis, G.E., Pintel, D.J., 2002. Characterization of the transcription profile of adeno-associated virus type 5 reveals a number of unique features compared to previously characterized adeno-associated viruses. J. Virol. 76, 12435–12447.

Rabinowitz, J.E., Rolling, F., Li, C., Conrath, H., Xiao, W., Xiao, X., Samulski, R.J., 2002. Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. J. Virol. 76, 791–801.

Rutledge, E.A., Halbert, C.L., Russell, D.W., 1998. Infectious clones and vectors derived from adeno-associated virus (AAV) serotypes other than AAV type 2. J. Virol. 72, 309–319.

Samulski, R.J., Berns, K.I., Tan, M., Muzyczka, N., 1982. Cloning of adeno-associated virus into pBR322: rescue of intact virus from the recombinant plasmid in human cells. Proc. Natl. Acad. Sci. USA 79, 2077–2081.

Senapathy, P., Carter, B., 1984. Molecular cloning of adeno-associated virus variant genomes and generation of infectious virus by recombination in mammalian cells. J. Biol. Chem. 259, 4661–4666.

Walters, R.W., Yi, S.M., Keshavjee, S., Brown, K.E., Welsh, M.J., Chiorini, J.A., Zabner, J., 2001. Binding of adeno-associated virus type 5 to 2,3-linked sialic acid is required for gene transfer. J. Biol. Chem. 276, 20610–20616.

Wang, X.-S., Qing, K., Ponnazhagan, S., Srivatava, A., 1997. Adeno-associated virus type 2 DNA replication in vivo: mutational analysis of the D sequence in viral inverted terminal repeats. J. Virol. 71, 3077–3082.

Xiao, X., Xiao, W., Li, J., Samulski, J., 1997. A novel 165-base-pair terminal repeat sequence is the sole *cis* requirement for the adeno-associated virus life cycle. J. Virol. 71, 941–948.

Xiao, W., Chirmule, N., Berta, S.C., McCullough, B., Gao, G., Wilson, J.M., 1999. Gene therapy vectors based on adeno-associated virus type 1. J. Virol. 73, 3994–4003.

Zabner, J., Seiler, M., Walters, R., Kotin, R.M., Fulgeras, W., Davidson, B.L., Chiorini, J.A., 2000. Adeno-associated virus type 5 (AAV5) but not AAV2 binds to the apical surfaces of airway epithelial and facilitates gene transfer. J. Virol. 74, 3852–3858.

Zolotukhin, S., Potter, M., Zolotukhin, I., Sakai, Y., Loiler, S., Fraites, T.J. Jr., Chiodo, V.A., Phillipsberg, T., Muzyczka, N., Hauswirth, W.W., Flotte, T.R., Byrne, B., Snyder, R.O., 2002. Production and purification of serotype 1, 2, and 5 recombinant adeno-associated viral vectors. Methods 28, 158–167.